An assessment of the modulation of the population dynamics of pathogenic Fusarium oxysporum f. sp. lycopersici in the tomato rhizosphere by means of the application of Bacillus subtilis QST 713, Trichoderma sp. TW2 and two composts

Research paper

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Running head: Biological Control of Tomato Fusarium Wilt with BCAs and composts

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Abstract

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol), results in considerable yield losses for tomato crops throughout the world. In this study, experiments were carried out *"in situ"* over two consecutive years to evaluate the efficiency of two biological control agents (BCAs) (*Bacillus subtilis* QST713 and *Trichoderma* sp. TW2) and two composts in controlling the disease. In this context, the quantitative polymerase chain reaction was used along with soil chemical parameters to study the general effects of treatments on the severity of the disease and on the non-target microbial populations residing in the studied rhizosphere and bulk soil. The ecological fitness of the BCAs was also evaluated. Furthermore, as BCAs produce elicitors which may activate plant defense reactions, particular attention was paid to the induction of pathogenesis-related genes (PR) in the roots of tomato plants.

The preventative nursery application of all three types of biocontrol agents, that is, *Bacillus subtilis* QST713, *Trichoderma* sp. TW2 and compost, as separate treatments, induced a significant reduction in the disease, compared to the untreated control, and reduced tomato Fusarium wilt by 70%. This result was confirmed by the significant negative correlations between the abundance of biological control agents and the severity of the disease. In general, the BCA and compost treatments did not induce a negative effect on the non-target microbial communities. The transcript levels coding for the studied pathogenesis-related (PR) genes were always higher in the presence of Fol on its own (untreated control) for all genes considered. However, the accumulation of transcripts in the tomato roots was different, depending on the treatment. An important level of disease reduction was shown by a decrease in Fol abundance, together with a greater abundance of the inoculated BCA populations and an accumulation of transcripts encoding PR genes.

In short, the results of this study reinforce the concept of the sustainability of treatments based on biological control agents and composts for the management of tomato Fusarium wilt.

Keywords: *Fusarium oxysporum* f.sp. *lycopersici* (Fol); BCAs; disease suppression; pathogenesis related genes (PR)

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is among the world's most important vegetables, with a global annual yield of approximately 160 million tons for all types of tomatoes (FAO, see the statistical database [FAOSTAT], 2014; available on: http://fatstat.fao.org). According to Lopes and Ávila (2005), about 200 biotic or abiotic diseases have been reported on tomato throughout the world. *Fusarium oxysporum* f. sp. *lycopersici* (Fol) is a soil-borne pathogen that causes the Fusarium wilt of tomato, which results in severe production losses (Gale et al., 2003; Huang et al., 2012; McGovern, 2015).

The eradication of soil-borne pathogens (including Fol) is generally based on an integrated management programme, consisting of chemical soil fumigation and the use of resistant cultivars when available (McGovern, 2015), but also on the use of organic amendments (Dukare et al., 2011; Pugliese et al., 2008; 2010) and biocontrol agents (BCAs) (Muslim et al., 2003; Gowtham et al., 2016; Huang et al., 2012; Jangir et al., 2018). *Bacillus* spp. and *Trichoderma* spp., which are available as commercial and experimental products, are among the most frequently used BCAs today (Jacobsen et al., 2004; Lorito and Woo, 2015). They are abundant in soil and have been investigated for their potential application as BCAs against several plant pathogens, including Fol (Cotxarrera et al., 2002; de Medeiros et al., 2017; Ghazalibiglar et al., 2016; Lorito et al., 2010; Zhao et al., 2014). The combination of BCAs with organic amendments (e.g., compost, manure, plant waste) has also been studied to control soil-borne plant pathogens (Borrero et al., 2004, 2012; Cotxarrera et al., 2002; Pugliese et al., 2011).

BCA activities include the secretion of secondary metabolites (such as cyclic lipopeptides and volatile organic compounds), and competition for nutrients and parasitism (Kim et al., 2015; Vitullo et al., 2012). In addition to these antagonistic effects on plant pathogens, BCAs produce elicitors that activate plant defence reactions (Walters and Daniell, 2007), such as the induction of pathogenesis-related (PR) genes (Edreva, 2005) encoding chitinase and β-1,3-glucanases. These two groups of PR proteins have attracted considerable interest because of their *"in vitro"* and *"in*

vivo" inhibitory activity against fungi (Van den Elzen et al., 1993; Bargabus et al., 2002). In addition, PR proteins (glucanases and chitinase) have been seen to increase concomitantly with the development of the disease (Rep et al., 2002). For example, it has been acknowledged that the treatment of sugar beet with *Bacillus mycoides* induces the expression of PR genes such as β-1,3-glucanase (Bargabus et al., 2002). As far as the inoculation of tomato plants with *F. oxysporum* f. sp. *Lycopersici* is concerned, increases in chitinase, β-1,3-glucanase, and β-1,4-glucosidase activities have been observed to be lower in resistant cultivars than in susceptible ones (Ferraris et al., 1987). However, relatively little is known about the effect of BCA and compost as pre-planting treatments on the accumulation status of PR genes in plants at the end of the cropping cycle in the correspondences with the peak of the disease (Borrero et al., 2004).

BCAs are known to colonise the rhizosphere, thereby indirectly affecting the microbial processes that are essential for the general functioning of the soil ecosystem. This rhizosphere colonization includes effects on the resident microorganisms and in consequence on carbon, nitrogen and phosphorus cycling (Bonanomi et al., 2010; Gupta et al., 2012). This may result in adverse effects on crop yield and health. Hence, there is a need to study the potential side - effects of the applied treatments on the indigenous rhizosphere and soil microbial populations (Bonanomi et al., 2018).

The main objectives of this study were: i) to evaluate the effect of two BCAs and two composts on the severity of the fusarium wilt of tomato and on the non-target rhizosphere and bulk soil microbial communities in the tomato - *Fusarium oxysporum* f.sp. *lycopersici* (Fol) pathosystem, in a naturally infested soil; ii) to obtain new insights into the induction of pathogenesis-related (PR) genes in pre-planting treated tomato roots at the end of the tomato crop cycle, which usually coincides with the highest disease incidence. Therefore, the differential expression of genes encoding *GluA* (acidic extracellular b-1,3-glucanase), *GluB* (basic intracellular b-1,3-glucanase), *Chi3* (acidic extracellular chitinase), *Chi9* (basic intracellular chitinase) and *PR-1a* was studied.

2. Materials and Methods

2.1. Layout of the experiments

Two experiments (2016 and 2017) were carried out in plastic tunnels on a commercial farm in Asti (Northern Italy), which had a history of several tomato cycles prior to the beginning of this study, in a sandy loam soil (sand : silt : clay 50 : 40 : 10 %, with neutral pH and 1.5 % organic matter), naturally infested with Fol.

The experimental trials were carried out over two consecutive years, 2016 and 2017, in order to test the efficacy of pre-planting soil treatments with a commercially available formulation of *Bacillus subtilis* QST713 - (SM, Serenade Max, Bayer Crop Science, Italy) and an experimental BCA (*Trichoderma* sp., TW2, AgriNewTech, Italy) (Table 1) against Fol. In addition, two commercial composts that is Ant's Compost V and Ant's Compost M; AgriNewTech, Italy, produced from green wastes in a dynamic industrial treatment system, were used (Table 1). Ant's Compost M is in fact Ant's Compost V inoculated with *Trichoderma* sp. TW2. An untreated control was used to monitor Fusarium wilt development. Summarised details of these treatments can be found in Table 1.

Tomato seeds (cv. Ingrid, Seminis) were sown in 40-plug trays (53 x 42 cm; plug 10x10 cm, 4l peat capacity) filled with a peat mixture substrate.

Four soil treatments with BCAs were carry out on the plug tray between sowing and transplanting in a greenhouse in a commercial nursery (Table 1). The BCAs were applied by spraying them onto the soil surface in a high volume of water (400 ml/tray) using a 1 l capacity hand sprayer. The application times were: 37 days after sowing, which was defined as T1, for the first treatment, 44 days after sowing, defined as T2, for the second treatment, 51 days after sowing, defined as T3, for the third treatment and 58 days after sowing, defined as T4, for the fourth treatment. The compost products were applied twice: they were first mixed with the substrate at sowing, and this combination was defined as T0, and they were then mixed with the soil one week before

transplanting at T3, that is, 51 days after sowing, according to dosages and application times reported in Table 1.

The tomato seedlings at the fourth true leaf stage were transplanted (T58) into naturally Fol infested soil at a density of 2.5 plants/m^2, drip irrigated and then grown according to the cultural practices adopted by commercial growers in the region. The plots were arranged in a completely randomised block design, with four replicates per treatment. The plants were monitored every 10-15 days to determine the development of symptoms. Disease severity (DS) data were recorded, starting from the appearance of the first symptoms (yellow leaves and reduced growth). The final disease rating was carried out 128 days after transplanting, on 16-20 plants per treatment by dissecting each plant. For this purpose, a DS scale was created as follows: 0 = healthy plant, 25% = initial leaf chlorosis, 50% = severe leaf chlorosis and initial symptoms of wilting during the hottest hours of the day, 75% = severe wilting and severe symptoms of leaf chlorosis; 100% = plant totally wilted, leaves completely necrotic (Borrero et al., 2004; Srinivasan et al., 2009).

2.2. Rhizosphere and bulk soil sampling

The effect of the two BCAs and composts on indigenous microbial communities was studied at the rhizosphere and bulk soil levels by sampling at the end of the trials (2016 and 2017), as described by Cucu et al., (2018). In brief, rhizosphere and bulk soil were collected, with three biological replicates per treatment. Each replicate consisted of five sub-replicates, which were pooled together. The replicate samples were collected in a W - shaped sampling pattern from each plot throughout the field, in order to ensure a good bulk soil homogeneity. The dry weight of the roots was also determined.

The fresh rhizosphere and bulk soil samples were accurately homogenized separately, sieved through a 2 mm sieve and stored at 4°C. All the samples were split for further geochemical analysis: humidity, pH, total N (TN), inorganic N as nitrate (NO_3^-) and ammonium (NH_4^+),

dissolved organic carbon (DOC), total phosphorus (Pt) and available phosphorus (Pav), as well as for molecular investigations.

2.3. Molecular analyses

2.3.1. Rhizosphere and bulk soil DNA extraction

A NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used, according to the manufacturer's instructions, for the rhizosphere and bulk soil genomic DNA extraction. The extraction was conducted on fresh samples (250-500 mg of sample material). The quantity and purity of the DNA were measured spectrophotometrically using a NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA).

2.3.2 Microbial abundance (quantitative PCR (qPCR) assays)

The abundance of the bacterial and archaeal 16S rRNA genes, fungal 18S rRNA genes and of three functional genes, (i.e., bacterial ammonia monooxygenase *amoA* - AOB, archaeal *amoA* - AOA and fungal chitinase - *chiA* was determined by means of qPCR, using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) for the rhizosphere and bulk soil DNA samples. In addition, the abundance of Fol, *Bacillus* spp. and *Trichoderma* spp. was also investigated. A description of the primer sets and amplification details is given in Table 2.

Amplicons were generated from each target gene for the standard preparation, purified (Invisorb Fragment CleanUp, Stratec Molecular GmbH, Berlin, Germany) and ligated in a Strata-Clone PCR cloning vector pSC-A (Strataclone PCR Cloning Kit, Agilent Technologies Inc.); the ligation products were then transformed using StrataClone SoloPack competent cells (Agilent Technologies Inc.). The specificity of the clones used as qPCR standards was checked, via sequencing, at LGC Genomics GmbH (Berlin, Germany), and through BLAST analysis. Plasmid DNA was isolated (GenElute™ Plasmid Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA) from standard clones and quantified as described above.

After the qPCR assay optimisation, the final volume of the qPCR reactions was established as 25 µl for the 16S and 18S rRNA genes, for the bacterial and archaeal *amoA*, and for the Fol and BCA quantification, while it was 20 µl for *chiaA*. All the qPCR assays were performed using 10 ng DNA as a template, except for the bacterial 16S rRNA gene, for which 5 ng DNA was used (Cucu et al., 2017; 2018). The reaction mixtures contained a 1x Power SYBR green master mix (Applied Biosystems), with 0.12 µM of each oligonucleotide (Table 2) for the total bacteria, archaea, fungi and Fol, 0.32 µM for the AOB, AOA and BCAs and 0.4 µM for *chiA*.

All the considered genes were quantified in triplicate across the plates, while standards were run in duplicate in 10-fold serial dilutions. The amplification efficiency ranged from 96%, (archaeal 16S rRNA, fungal 18S rRNA, BCAs and their functional genes) to 103% (bacterial 16S rRNA). The amplification efficiency of the nitrifiers was 95.3% and 99.1% for AOB and AOA, respectively. R^2 was always ≥ 0.98. Melting curves of the amplicons were generated to ensure that the fluorescence signals originated from specific amplicons and not from primer dimers or other artifacts. This was confirmed by checking the amplification products on 1% agarose gel. Gene copy numbers were calculated with StepOne™ software, version 2.2 (Applied Biosystems). The data were normalised and presented in figures as copies g^{-1} dry soil.

2.3.3 Race identification of *Fusarium oxysporum* f. sp. *lycopersici* from the experimental field

Real time PCR was used to identify Fol from the experimental field. Four primer/probe sets were used to identify the tomato wilt pathogen race, on the basis of rDNA -intergenic spacer and avirulence genes. Real - time PCR trials were conducted using genomic DNA from mycelia isolated from the experimental site and soil DNA. Reaction conditions were established according to the protocol described by Inami et al., (2010).

2.3.4 RNA extraction and real-time RT-PCR gene expression analysis

In order to study the differential expression of the selected genes in plants pre-planting treated with biocontrol agents and compost, the real time RT-PCR assay was used on RNA samples from roots collected at the end of the tomato crop cycle, which had been conserved at -80˚C. The total RNA was extracted by means of an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) using 0.1 g of tomato roots. The RNA was DNase treated using TURBO DNase (Ambion™, Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's protocol, and then reverse transcribed (500 ng total RNA) using a high capacity cDNA reverse transcription kit (Applied Biosystems). The actin gene was used as a reference gene. The primers for the *LeChi3*, *LeChi9*, *TomB13GluA*, *TomB13GluB*, and *LePr1a* target genes were used according to Aimé et al., (2008). Real-time PCR reactions were carried out with 25 ng of cDNA, 500 nM of each primer, 10 ml of the 1x Power SYBR green master mix (Applied Biosystems) and RNase-free water in a final volume of 20 ml. cDNA was replaced by RNase free water in the negative control. A programme consisting of 15 min at 95˚C, followed by 40 denaturation cycles for 15 s at 95˚C, annealing for 30 s at 58˚C and extension for 30 s at 72˚C was used for the real-time PCR, at the end of which fluorescence was measured. Real-time PCR reactions were carried out in duplicate for each sample. Primer titration and dissociation experiments were performed to confirm there was no formation of primer dimers or false amplicons that could interfere with the results. After the real-time PCR experiment, the Ct number was extracted for both the reference gene and the target gene considering an auto baseline and a manual threshold. Gene expression levels (relative to the actin gene) were calculated for each cDNA sample using the following equation: relative gene / actin = (E gene - Ct gene) / (E actin - Ct actin) ratio.

2.4. Chemical properties of the rhizosphere and bulk soil samples

The pH values were measured in water suspensions at a solid:liquid ratio of 1:2.5. The total nitrogen (TN) was quantified using a Leco Tru Spect CN automatic analyser. The dissolved organic

carbon (DOC) was determined after sample acidification in a TOC/TN analyser (Multi NC 2100S, Analytic Jena GmbH, Jena, Germany). Ammonium (NH_4^+) and nitrate (NO_3^-) were measured colorimetrically, by means of a continuous flow auto-analyser (Alliance Evolution II), using standard colorimetric techniques. The total phosphorus (Pt) was determined by means of 'ICP Varian mod. Liberty LR', after microwave digestion with hydrogen peroxide, hydrochloric acid and nitric acid, filtration and dilution. The available P (Pav) was extracted using sodium bicarbonate and determined by means of the molybdenum blue method (Olsen et al., 1954), modified for continuous flow colorimetric analysis (Alliance Evolution II).

2.5. Statistical analyses

The data on gene abundance, on the chemical properties of the soil and on the disease index were subjected to a linear mixed model, with time considered as the random variable, using R software (Software R 3.0.1, R foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). The effects of two different BCA and compost applications on the abundance of the studied genes, on the chemical properties of the soil and on disease severity were evaluated. All the data were subjected to a Levene test to check for the homogeneity of variance, and normality was tested on the residuals using the Shapiro - Wilk test; when not normally distributed, data from disease severity (DS) were arcsin transformed, while the data from microorganism abundance were log-transformed and normality was checked again. The means were separated by means of the Bonferroni test. The statistical analysis included treatment, year, treatment × year and rhizosphere × bulk soil.

Pearson's linear correlation coefficients were calculated to assess the relationships between microbial abundance and disease severity as well as between BCA and Fol abundance and the soil chemical properties.

3. Results

3.1. Disease severity

In 2016, in the presence of a low level of disease severity (DS 29.7%) in the untreated control - C tomato plots, the two tested BCAs and composts provided a disease reduction of 89.5 to 93%. The green compost - CV provided statistically similar results to those of the compost - CM, which was enriched with *Trichoderma* sp. TW2 (a disease reduction of 93% and 64%, respectively). However, no significant differences were observed between treatments. In 2017, a disease severity of 39.9% was observed in the untreated tomato control plants, but all the treatments significantly reduced Fusarium wilt symptoms as much as 50%, and statistically similar results between treatments were obtained (Table 3).

3.2. Microbial abundance

3.2a Race identification and the abundance of soil-borne F. oxysporum f.sp. *lycopersici* (Fol)

The amplification reaction for the Fol race identification was positive for both the P1 and R1 Race - 1 specific sets (Inami et al., 2010), on the basis of the rDNA-IGS and the avirulence genes, respectively (data not shown).

In general, Fol abundance was significantly higher in the bulk soil than in the rhizosphere soil, P(F) Rhizo*Bulk soil = 0.003. All the treatments resulted in a significant pathogen reduction, compared to the untreated control - C, in both the rhizosphere and bulk soil at the end of both trials (2016 and 2017) (Figure 1). The treatment × year interaction was not significant.

3.2b Total microbial community abundance

At the end of both trials (2016 and 2017), the bacterial, archaeal and fungal communities showed a notable homogeneity, with no significant treatment × year interaction. Therefore, the data were presented as the average of all the values of both trials.

Overall, at the end of both trials, the bacterial 16S rRNA abundance was significantly higher at the bulk soil level than at the rhizosphere level (Table 4). A higher bacterial abundance was observed in rhizosphere soil samples after the *Trichoderma* sp. TW2 - TW2 treatment, than in the untreated control -C, which was significantly similar to the green compost - CV treatment. On the other hand, the highest bacterial abundance at the soil level was observed after the inoculated compost - CM treatment, while the lowest was observed after the *B. subtilis* treatment. The archaeal 16S rRNA gene abundance in the bulk soil samples was more stable and was not influenced by the treatments. However, the green compost - CV, green compost - CM+TW2, and *B. subtilis* treatments resulted in an elevated archaeal presence, compared to the untreated control - C in the rhizosphere soil samples (Table 4). The fungal 18S rRNA abundance was significantly higher in the rhizosphere samples than in the bulk soil ones (Table 4). A significantly higher fungal abundance was observed in the rhizosphere after the inoculated compost - CM, *Trichoderma* sp. TW2 - TW2 treatments as well as in the untreated control - C. *B. subtilis* and green compost CV significantly reduced the total fungal population, while the compost - CM, *Trichoderma* sp. TW2 - TW2 treatments did not affect it. At the bulk soil level, the highest fungal abundance was observed after the green compost - CV treatment, while the untreated control - C was statistically similar to the *Trichoderma* sp. TW2 - TW2 treatment. *B. subtilis* and green compost CM+TW2 significantly reduced the total fungal population.

3.2c Functional gene abundance

In general, the ammonia-oxidising bacterial (AOB) gene abundance was not significantly influenced by the treatments, compared to the untreated control, and was significantly more abundant in the rhizosphere soil than in the bulk soil samples. The highest abundance of AOB was observed in the untreated control - C of the bulk soil and the lowest value was observed for the untreated control - C of the rhizosphere samples (Table 5). In general, the ammonia-oxidising archaeal (AOA) gene abundance was significantly more abundant in the bulk soil samples than in

335 the rhizosphere soil ones (Table 5). The highest abundance in the rhizosphere samples was observed
336 after the compost - CM treatment, while the highest abundance in the bulk soil samples was
337 observed after the compost - CV treatment (Table 5).
338
339 3.2d BCA abundance
340 Overall, at the end of both trials, an increase in the indigenous populations of *Bacillus* spp. and
341 *Trichoderma* spp. was observed for all the treatments, compared to the untreated control - C. No
342 significant treatment × year interaction was observed. In general, the abundance of *Bacillus* spp.
343 was significantly higher for the *B. subtilis* - SM treatment than for the untreated control - C and for
344 all the other treatments in both the rhizosphere and bulk soil. However, both compost treatments
345 resulted in an increased resident *Bacillus* abundance in the rhizosphere and bulk soils after both
346 trials. Nevertheless, the *Trichoderma* sp. TW2 - TW2 - treatment showed similar abundance to
347 those of the untreated control - C (Table 6).
348 The inoculation with *Trichoderma* sp. significantly increased the overall *Trichoderma* spp.
349 community in both the rhizosphere and in the bulk soils, compared to the untreated control - C. The
350 highest abundance of *Trichoderm*a spp. was observed in the rhizosphere after the compost - CM
351 treatment and in the bulk soil after the *Trichoderma* sp. TW2 - TW2 treatment (Table 6). *B. subtilis*
352 - SM resulted in a somewhat lower abundance than the *Trichoderma* sp. - TW2 treatment.
353 In general, the *chiA* gene (*Trichoderma* based chitinase) abundance was significantly influenced by
354 the treatments, compared to the untreated control - C (Table 7). All the treatments at the rhizosphere
355 soil level resulted in significant differences between the *chiA* gene abundances. The abundance of
356 the *chiA* gene was higher in the rhizosphere soil samples for the *Trichoderma* sp. TW2 and the
357 compost - CM treatments and lower and significantly similar to the untreated control - C after the *B.*
358 *subtilis* - SM treatment. All the treatments at the bulk soil level also resulted in a higher *chiA*
359 abundance than the untreated control - C. The highest *chiA* gene abundance at the bulk soil level
360 was observed after the green compost - CV treatment.

3.2e Gene expression - analysis with RT-PCR

The levels of the transcripts encoding the studied pathogenesis-related (PR) genes were always higher for the presence of Fol on its own (untreated control - C samples) for all the considered genes. The transcript accumulation in the tomato roots differed according to the treatments (Figure 2); the transcript level profiles encoding *Chi3* and *Chi9* were rather similar, and were higher in the *Trichoderma* sp. based treatments than the treatments with *B. subtilis* - SM and green compost - CV. Overall, the expression level of *GluA* was lower than the expression level of *GluB.* The *B. subtilis* - SM and both compost treatments showed a slightly higher expression of *GluA* than the *Trichoderma* sp. TW2 - TW2 inoculation on its own. The *GluB* gene was expressed more in the *B. subtilis* - SM inoculated compost and *Trichoderma* sp. TW2 - TW2 treatments. The expression level of the PR-1 gene was very low for all the treatments.

3.3. Chemical properties

The pH was generally significantly lower ($p < 0.05$) at the rhizosphere level than at the bulk soil level for both trials, with values ranging from 7.43 to 7.60 for the rhizosphere and between 7.95 and 8.18 for the bulk soil samples. The concentrations of TN, NH_4^+, NO_3^- and DOC were generally significantly higher in the rhizosphere samples than in the bulk soil ones. The untreated control - C was characterised by higher NH_4^+, NO_3^- and DOC concentrations than all the treatments at a bulk level (Table S1). No significant treatment × year interaction was observed.

The dry weight of the roots was not significantly different between the SM, CM and CV treatments. The highest dry weight was observed after the TW2 treatment, while the lowest was observed in the untreated control - C (Figure S1).

3.5. Correlations between the total microbial communities (16S bacteria, 16S archaea and 18S fungi), the functional genes (fungal *chiA* gene, bacterial and archaeal *amoA* genes,), the BCAs (*Bacillus, Trichoderma*) and Fol abundances, and DS.

In general, the correlations were negative, with r coefficients ranging from -0.3 to -0.7 ($p < 0.001$) at the rhizosphere level and from -0.3 to -0.8 ($p < 0.001$) at the bulk soil level (Table 8 and Table 9, respectively).

4. Discussion

In the present study, pre-planting treatments with BCAs (*Bacillus subtilis* and *Trichoderma* sp. - TW2) and two composts were used to control tomato Fusarium wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Race 1) in naturally infested soil. The evaluation was based on the two-year effect of these treatments on the disease incidence, PR gene expression and on the rhizosphere/soil microflora and chemical properties. The risk assessment of the BCAs and compost utilised within the frame of this study pointed out the impact these treatments had on the pathogen population and disease control corroborated with the PR gene expression status, as well as on the rhizosphere and bulk soil microbial community dynamics. This impact is important as the resident microbes may colonise similar niches as the newly introduced BCA microorganisms and thus compete for similar resources in the rhizosphere and soil environment (Winding et al., 2004).

4.1. Disease severity, and F. oxysporum f. sp. *lycopersici* (Fol) *abundance*

The preventative application of two BCAs and composts, starting from the nursery, significantly reduced Fol Race 1 abundance, compared to the untreated control. These findings were in accord with the results of previous studies that showed that *Fusarium* abundance at the rhizosphere level of different hosts, such as cucumber, banana and lettuce, was reduced after BCA treatments (Qiu et al., 2012; Shen et al., 2015; Fu et al., 2017). In the present study, Race 1 of Fol abundance was low after the treatment with the enriched compost (CM), thus indicating that an effective Fusarium wilt control may be achieved on tomato with organic amendments, as previously pointed out (Borrero et al., 2004; Cotxarerra et al., 2002). The compost utilisation probably resulted in changes in the

activity, density and structure of the non - target microbial community in the soil, thus enhancing competition with the pathogen for space and nutrients, such as carbon (C) and nitrogen (N). This may have resulted in a reduction in the Fol abundance and its potential activity, as already reported by Bonilla et al., (2012), Cucu et al., (2018), Larkin et al., (2015).

4.2. Plant response to Fol infection

Plants usually have a complex defence system against pathogens that are well known as stress factors, including different types of stress proteins with putative protective functions (Brown et al., 2017). A high accumulation of transcripts encoding the studied PR genes in the plant roots from the untreated control was observed in this study. This may be correlated to the colonisation of the tomato vessels by Fol, as previously reported by Olivain and Alabouvette, (1999). The here presented results are in line with those of Aime´ et al., (2008), who found a higher PR gene expression in tomato plants inoculated with a pathogenic strain, than in plants inoculated with a non-pathogenic strain of *F. oxysporum*. Moreover, the pre-inoculation of plants with *Trichoderma* sp. resulted in an even higher expression of PR genes encoding chitinase, thus indicating that *Trichoderma* spp. induced a potentiated status in the plant that enabled it to be more resistant to subsequent pathogen infection, as was also observed by Shoresh et al., (2005). This hypothesis is in line with that of Shoresh et al., 2005 and Yedidia et al., 2003, who found that, in a *T. asperellum* - cucumber system, PR protein chitinase, β-1,3-glucanase and peroxidase were induced by *Trichoderma*. Jangir et al., (2018) showed that *Bacillus* sp. produced chitinase and β-1, 3-glucanase that might play a role in the digestion of the pathogen's hyphae as carbon used than as energy sources. Bargabus et al., (2002) showed that the treatment of sugar beet with *Bacillus mycoides* induced the expression of PR genes that is new isoforms of β-1,3-glucanase. The potential production of PR-proteins with chitinase and β-1,3- glucanase activity is presumably part of the hypersensitive defence mechanism of tomato plants and may be responsible for the induction of the resistance developed by the plant after treatment with the antagonists and infection with Fol.

4.3. Ecological feedback of the applied treatments and their effect on the total indigenous prokaryotes

Rhizosphere microbial communities play important roles in plant health and disease prevention (Bonanomi et al., 2018; Dudenhöffer et al., 2016). Analysing the abundance of the BCA - like microorganisms, introduced into the soil as strains or through the compost treatments, showed a very good feedback of the targeted populations (e.g., *Bacillus* spp. and *Trichoderma* spp.) as well as excellent efficiency, as highlighted by the strong negative correlations with disease severity.

The abundance of *Trichoderma* spp. showed different trends in all the treatments. A higher abundance of *Trichoderma* was detected in the rhizosphere of the inoculated compost treatment, but also after the inoculation of the single strain. It is well known that composts represent an optimal substrate for *Trichoderma* spp., as the organic matter composition and the associated biotic and abiotic conditions can in general influence *Trichoderma* activity (Vinale et al., 2008; Lorito and Woo, 2015). *Trichoderma* agents have been reported to be antagonistic to the *Fusarium* pathogens: *F. oxysporum* f. sp. *ciceris*, *F. solani*, *F. oxysporum* f. sp. *lycopersici* (Dubey et al., 2007; Rojo et al., 2007; Christopher et al., 2010). Gachomo and Kotchoni (2008) revealed that *Trichoderma* isolates displayed various extracellular enzyme activities, for example amylase, chitinase, pectinase, protease, lipase and cellulase activities, in order to compete with other microbes, and that the production of volatile compounds by *Trichoderma* species could inhibit the growth of pathogenic microorganisms. One of the main mechanisms of *Trichoderma* in the control of the soil-borne pathogen is due to the presence of genes encoding cell wall degrading enzymes (Lorito et al., 1998, Lorito and Woo, 2015). In this regard, the abundance of the *chiA* gene and its negative correlation with disease severity has confirmed the potential antagonistic activity of the inoculated *Trichoderma* strain TW2, but also of the inoculated compost - CM. This result has pointed out an enhanced nitrogen efficiency as a result of the improvement of the nitrogen reduction and assimilation mechanism, as also evidenced by Lorito et al., (2010) and Borrero et al., (2012). In addition, the competition for carbon and other growing factors at the rhizosphere level helped to

sustain the control of Fol by *Trichoderma*, as highlighted by the very good correlation with dissolved organic carbon at the rhizosphere and bulk soil levels as well as with the ammonium content at the rhizosphere level. These results support the results of Sivan and Chet (1989), who demonstrated that the competition for nutrients is the major mechanism used by *T. harzianum* to control *F. oxysporum* f. sp. *melonis*.

The *Bacillus* sp. treatment also resulted to be effective in reducing the disease. The abundance results highlighted the excellent feedback of *Bacillus* spp. from the rhizosphere and soil levels. The significant negative relationships ($p < 0.001$) between *Bacillus* spp. and disease severity pointed out the antagonistic function of *Bacillus* against Fol, which may be due to volatile compounds and siderofori production, as reported by Jangir et al., (2018) after having characterised antagonistic *Bacillus* sp., isolated from a tomato rhizosphere, and to its control mechanisms against Fol.

Although a significant suppression of Fusarium wilt was achieved when the BCAs were employed individually (i.e., SM, TW2), alone or together with compost (i.e., CM), it is possible to assume, in agreement with Baker (1990), that the effects that were observed on antagonistic microbial communities after BCA applications may be the key to a successful and integrated approach for the control of tomato Fusarium wilt. In addition, as reported by Vinale et al., (2008), the biotic components of the soil environment also have relevant effects on the activity of the used biocontrol agents.

Overall, the release and successful proliferation of BCAs in the studied soil did not negatively alter the abundance of the functionally relevant indigenous soil microorganisms. The here presented results support previous reports which were based on the analysis of microbial densities (Ghini et al., 2000; Gullino et al., 1995; Mezzalama et al., 1998). A higher total fungal abundance was observed in the rhizosphere than in the bulk soil. The increased fungal community that was observed for all the treatments and for the untreated control was probably caused by the elevated level of organic carbon (Table S1). This may be linked to a competitive potential, based on an increased root exudation, which favours rhizosphere colonisation by fungi, including Fol and

491 *Trichoderma* spp. This result has been corroborated by considering the root dry weight (Figure S1).
492 According to Griffiths and Philippot (2013), the results suggested a great tolerance and resilience
493 potential of the native fungal community towards invading microorganisms (BCAs). Similarly,
494 Edel-Hermann et al., (2009) and Savazzini et al., (2009) only observed transient community shifts
495 in indigenous microbial populations in response to inoculation with antagonistic *Fusarium*
496 *oxysporum* and *Trichoderma atroviride*. However, a difference among treatments was observed at
497 both rhizosphere and bulk soil level, as *B. subtilis* and compost resulted in a decrease of total fungal
498 abundance. This result highlighted on one hand the rhizosphere competence of *B. subtilis* which
499 probably had a quick take over of space and nutrients. On the other hand the capacity of plant to
500 select the beneficial microorganisms as well as the influence of soil characteristics are well known
501 to be keys of soil biomass density and composition (Chapparo et al., 2012) Compost and
502 formulations of compost with bacteria or fungi (e.g., *Trichoderma* W2) may also dictate
503 competition for nutrients and the predominance of some microbial groups to another (Pugliese et al.
504 2011).
505 A high resilience of the total archaeal community was observed at both the rhizosphere and the soil
506 level, compared to the total bacterial community. Bacterial abundance was lower at the rhizosphere
507 level than at the bulk soil level, probably as a result of increased competition with the fungal
508 counterparts, including Fol. Archaeal nitrifiers (AOA) were more abundant in the bulk soil samples
509 than in the rhizosphere samples, which may be a result of the lower organic carbon content of the
510 soil and the higher pH (Wessén et al., 2010; Bates et al., 2011). A negative correlation was found
511 between the abundance of AOA and Race 1 of Fol at a soil level, thus suggesting a direct effect of
512 archaeal nitrifiers on the pathogen. However, these speculative interpretations require further
513 research to clarify the mode of actions of AOA responses to the development of Fol in the soil of
514 tomatoes. Nevertheless, the higher AOA abundance in the soil samples indicated that the AOA had
515 probably adapted to the decomposition of recalcitrant organic matter, as already described by Cucu
516 et al. (2017). The same pattern was observed by Cucu et al., (2018) for a lettuce - Fusarium wilt

pathosystem. This highlights the importance of abiotic factors, such as pH and soil type, in driving not only the dynamics of prokaryotes, but also a higher level of suppressiveness towards *Fusarium* spp. in the soil environment with pH ~/8. This finding supports the findings of earlier studies which correlated soil suppressiveness to *Fusarium* spp. with such abiotic soil characteristics as the clay content and pH (Höper et al., 1995; Yergeau et al., 2010). The bacterial nitrifiers showed a higher abundance and, as a consequence, a higher resilience at the rhizosphere level than the archaeal counterparts. This result, as already pointed out by Wessén et al., (2010), points out that a low ammonia environment is the key factor that determines a niche separation of AOA and AOB in neutral soils.

The effect of *Bacillus* sp. and *Trichoderma* sp. on the nitrifiers was similar between treatments, thus suggesting that *Bacillus subtilis* and *Trichoderma* sp. TW2 may not have any negative ecological impact on other groups of microorganisms

Conclusions and outlook

The results have pointed out that the application of *Bacillus subtilis*, *Trichoderma* sp. TW2 and compost treatments is safe for the rhizosphere and soil resident non-target microbial communities as well as for the effective control of Fol on tomato. Moreover, the different response of the AOA population to various treatments at a rhizosphere and a bulk level could indicate their suitability as indicators to assess perturbations in soils, for example as the result of the introduction of new microorganisms which act as biocontrol agents in the soil environment. Nevertheless, for a better understanding of the impact that different treatments (e.g., biological or chemical) may have on *Fusarium oxysporum* f.sp. *lycopersici*, it is necessary to consider not only non-target soil microbial communities, but also different soil types characterised by different physical - chemical properties, as abiotic factors.

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Conflict of Interest

The authors declare that they have no conflict of interest. Massimo Pugliese declares he has a financial interest (shareholder) in the AgriNewTech company that provided some of the products tested in this study.

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Figure Captions

Figure 1 Abundance of *Fusarium oxysporum* f.sp. *lycopersici* (Fol) in the rhizosphere and bulk soil for different treatments and the untreated control (SM - *Bacillus subtilis* - Serenade Max; CM - Green compost plus *Trichoderma* sp. TW2; CV - green compost; TW2 - *Trichoderma* sp. TW2; C - untreated control) (n = 6, means±standard errors). Different letters above the bars indicate significant differences between treatments in the rhizosphere (uppercase letters) and bulk soil (lowercase letters); *indicate significant differences between rhizosphere and bulk soil

Figure 2 Real-time RT-PCR analysis of PR gene expression in the tomato roots for different treatments and for the untreated control. Actin was used as the reference gene. The error bars show standard deviations for triplicate assays.

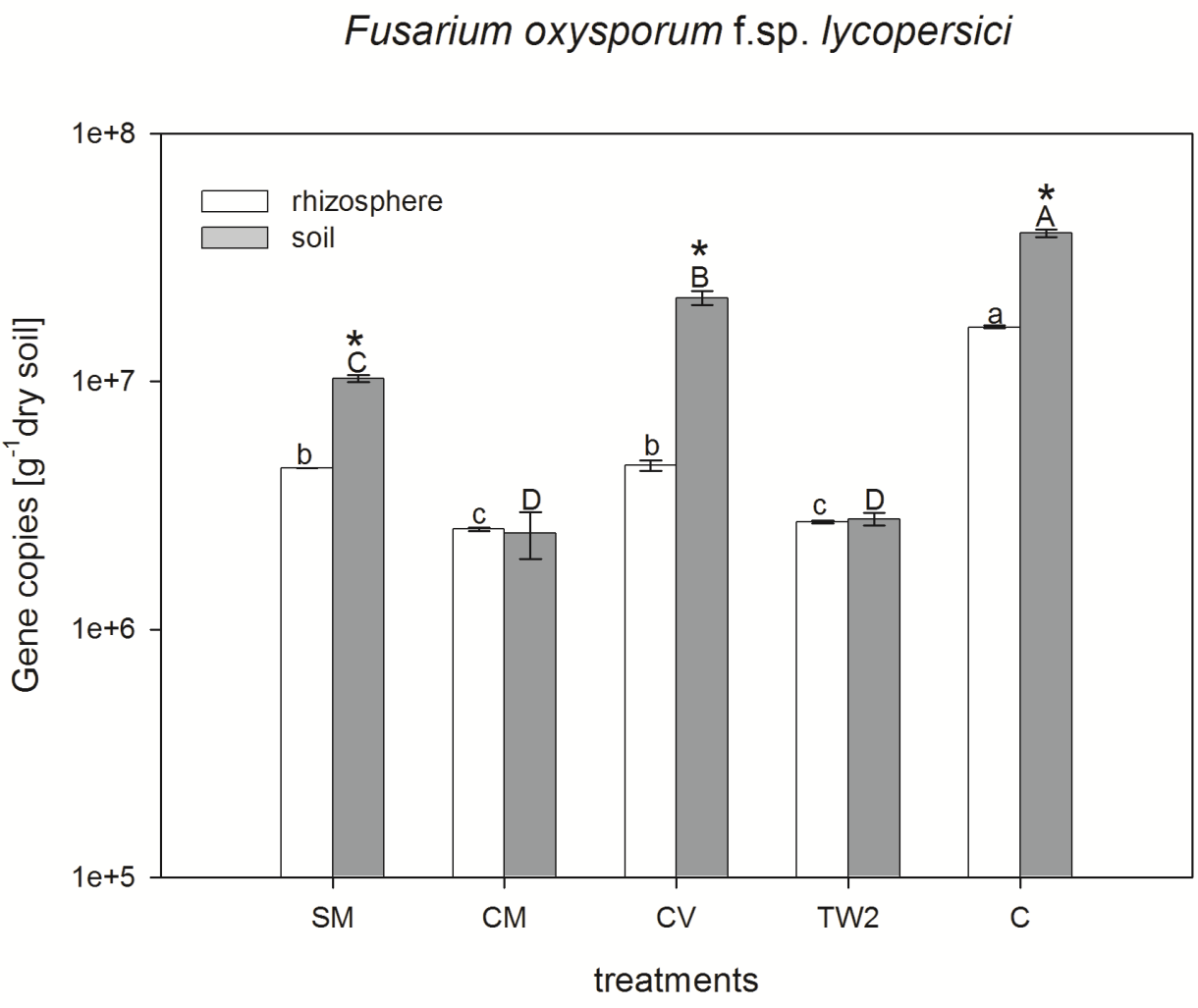
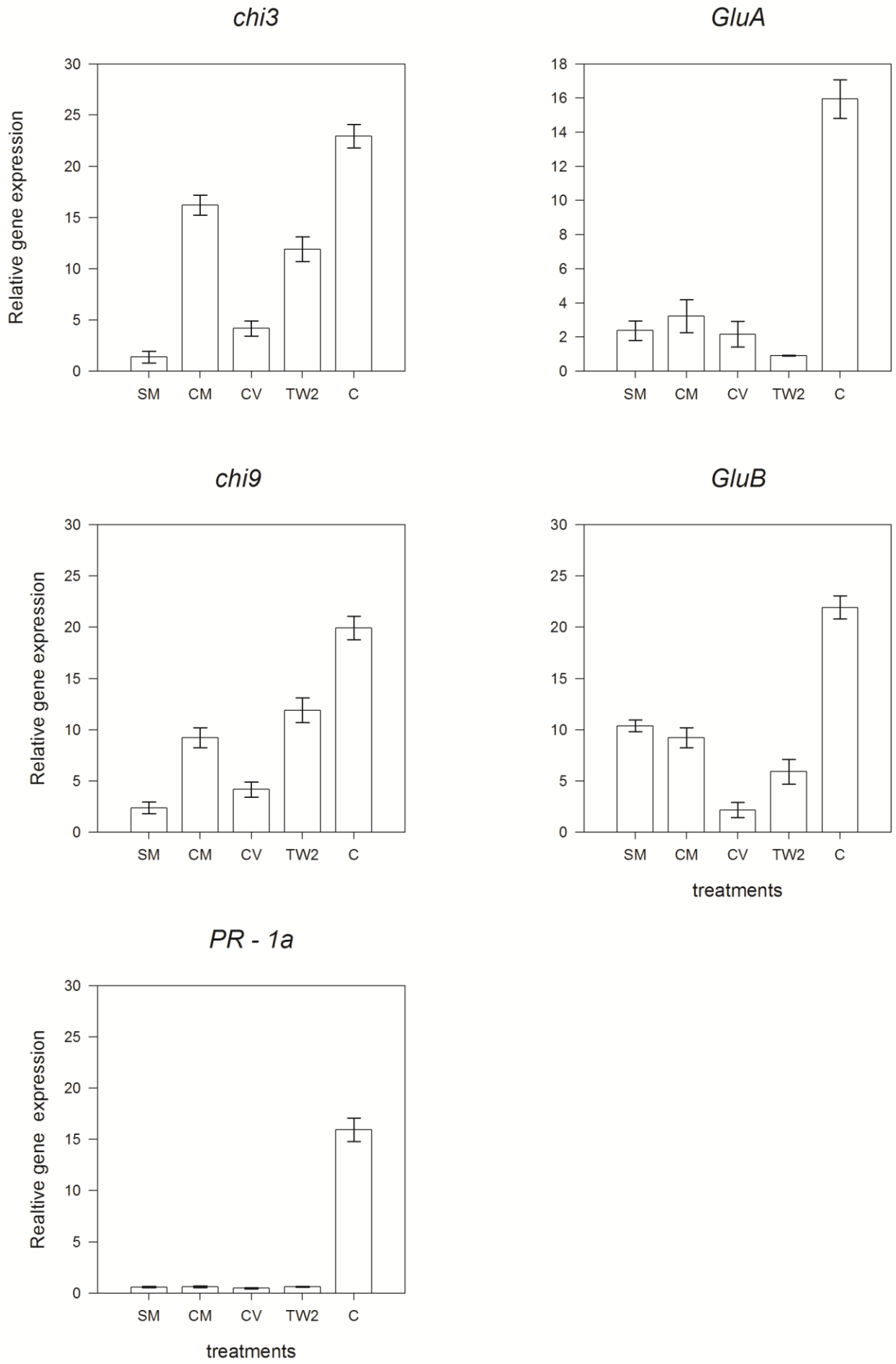


Figure 1


chi3
Relative gene expression
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treatments
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Figure 2

Tables

Table 1 General information on the trials and timing of the operations carried out in 2016 and 2017

Treatment	Microorganism/a.i.	Dosage	Tray treatment	Plot Treatment
Serenade max - SM	*Bacillus subtilis* QST 713	$2.9X10^{10}$ cells/L water	T37; T44; T51;T58*	-
ANT'S COMPOST M - CM	*Green compost + Trichoderma* sp. TW2	8 g/seedling;1kg/0.1m^3 of soil	T0	- T51
ANT'S COMPOST V - CV	*Green compost*	8 g/seedling; 1kg/0.1m^3 of soil	T0	- T51
Trichoderma sp. TW2 -TW2	*Trichoderma* sp. TW2	1x10^7 (conidia /ml)	T37; T44; T51;T58	
Untreated control - C	-	-		

*Treatments: T0 at sowing; T1, 37 days after sowing; T2, 44 days after sowing; T3: 51 days after sowing; T 4: 58 days after sowing and immediately before transplanting.

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845 Table 2 Description of the primer sets and amplification details used for the quantitative PCR.

Target group	Primer (reference)	Amplification details
Fusarium oxysporum f.sp. *lycopersici* (Fol)	FO1(Prashant et al., 2003) 5′-ACATACCACTTGTTGCCTCG-3′ FO2 (Prashant et al., 2003) 5′-CGCCAATCAATTTGAGGAACG-3′	40 cycles 95°C 15s, 60°C 60s, 72°C 45s
All bacteria (16S rRNA gene)	Eub338 (Lane 1991) 5′-ACTCCTACGGGAGGCAGCAGCAG-3′ Eub518 (Muyzer et al. 1993) 5′-ATTACCGCGGGCTGCTGG-3′	40 cycles 95°C 30s, 55°C 35s, 72°C 45s
All archaea (16S rRNA gene)	340F (Gantner et al., 2011) 5′-CCCTAYGGGGYGCASCAG-3′ 1000R (Gantner et al., 2011) 5′-GGCCATGCACYWCYTCTC-3′	40 cycles 95°C 30s, 57°C 30s, 72°C 30s
All fungi (18S rRNA gene)	FR1 (Vainio and Hantula, 2000) 5′-AIC CAT TCA ATC GGT AIT-3′ 390FF (Vainio and Hantula, 2000) 5′-CGA TAA CGA ACG AGA CCT-3′	45 cycles 95°C 30s, 50°C 30s, 70°C 60s
Ammonia oxidizing bacteria (AOB*)*	AmoA-1f (Rotthauwe et al. 1997) 5′-GGGGTTTCTACTGGTGGT-3′ AmoA-2r (Rotthauwe et al. 1997) 5′-CCCCTCKGSAAAGCCTTCTTC-3′	45 cycles 95°C 30s, 57°C 45s, 72°C 45s, 78°C 20s
Ammonia oxidizing archaea (AOA)	Arch-amoAf (Francis et al., 2005) 5′-STAATGGTCTGGCTTAGACG-3′ Arch-amoAr (Francis et al.,2005) 5′-GCGGCCATCCATCTGTATGT-3′	45cycles 95°C 30s, 53°C 45s, 72°C 45s, 78°C 20s
chiA gene	chiaxf (Cucu et al., 2018) 5′-ACCCTGCCGATGACACTCAG-3′ chiaxr (Cucu et al., 2018) 5′-GGCAGCGATGGAGAGAAGGA-3′	35cycles 95°C 15s, 59.9°C 30s, 70°C 30s
Bacillus subtilis	Forward *B. subtilis* (Gao et al., 2011) 5′-TCGCGGTTTCGCTGCCCTTT-3′ Reverse *B. subtilis* (Gao et al., 2011) 5′-AAGTCCCGCAACGAGCGCAA-3′	40 cycles 95°C 30s, 60°C 60s, 80°C 10s
Trichoderma spp.	uTf (Hagn et al., 2007) 5′-AACGTTACCAAACTGTTG-3′ uTr (Hagn et al., 2007) 5′-AAGTTCAGCGGGTATTCCT-3′	35 cycles 95 °C 30 s, 55.5 °C 30 s, 72 °C 30 s

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Table 3 Effect of the preventative soil treatments with BCAs and compost on disease severity at the end of trials 1 and 2, in 2016 and 2017, respectively

Treatment	Disease severity** %			
	2016		2017	
Bacillus subtilis QST 713 - SM	6.3	±3.8[a]	19.5	±3.0[a]
Green compost + Trichoderma sp. TW2 - CM	10.4	±5.2[a]	21.9	±2.8[a]
Green compost - CV	1.9	±1.9[a]	14.5	±2.6[a]
Trichoderma sp. TW2 - TW2	3.1	±3.1[a]	16.0	±1.3[a]
Untreated control - C	29.7	±2.3[b]	39.9	±2.4[b]

*Treatments: T0 at sowing; T1, 37 days after sowing; T2, 44 days after sowing; T3: 51 days after sowing; T 4: 58 days after sowing and immediately before transplanting.

**Sixteen plants/treatment were evaluated for disease severity using the following rating scale: 0 = healthy plant, 25 = initial leaf chlorosis, 50 = severe leaf chlorosis and initial symptoms of wilting during the hottest hours of the day, 75 = severe wilting and severe symptoms of leaf chlorosis; 100 = plant totally wilted, leaves completely necrotic.

883 Table 4 Abundance of 16SrRNA (all bacteria and archaea) and 18S rRNA (fungi) in the rhizosphere
884 and bulk soil after different treatments and in the untreated control
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Treatment	Rhizosphere soil	Bulk soil
	16S Bacteria (logcopy DNA $^{-1}$)	
Bacillus subtilis (SM)	10.686 c	10.963 d
Green compost+TW2 (CM)	10.755 b	11.287 a
Green compost (CV)	10.454 d	10.904 d
Trichoderma sp. TW2 (TW2)	10.903 a	11.191 b
Untreated control - C	10.477 d	11.077 c
P(F) Treat	*0.000*	
P (F) Year	*0.000*	
*P(F) Treat*Year*	*ns*	
*P(F) Rhizo*Bulk soil*	*0.000*	
	16S archaea (logcopy DNA $^{-1}$)	
Bacillus subtilis (SM)	7.728 a	7.995 a
Green compost+TW2 (CM)	7.748 a	7.982 a
Green compost (CV)	7.751 a	8.023 a
Trichoderma sp. TW2 (TW2)	7.592 b	7.974 a
Untreated control - C	7.549 b	7.988 a
P(F) Treat	*0.000*	
P (F) Year	*0.000*	
*P(F) Treat*Year*	*ns*	
*P(F) Rhizo*Bulk soil*	*0.002*	
	18S funghi (logcopy DNA $^{-1}$)	
Bacillus subtilis (SM)	9.669 c	8.955 b
Green compost+TW2 (CM)	10.111 a	8.927 b
Green compost (CV)	9.813 b	9.226 a
Trichoderma sp. TW2 (TW2)	10.000 a	8.631 c
Untreated control - C	9.979 a	8.542 c
P(F) Treat	*0.000*	
P (F) Year	*0.000*	
*P(F) Treat*Year*	*ns*	
*P(F) Rhizo*Bulk soil*	*0.000*	

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Table 5 Abundance of bacterial (AOB) and archaeal (AOA) *amoA* genes in the rhizosphere and bulk soil after different treatments and in the untreated control

Treatment	Rhizosphere soil		Bulk soil	
	AOB (logcopy DNA $^{-1}$)			
Bacillus subtilis (SM)	7.316	a	6.233	c
Green compost+TW2 (CM)	7.280	a	6.665	b
Green compost (CV)	7.321	a	6.686	b
Trichoderma sp. TW2 (TW2)	7.387	a	6.603	b
Untreated control - C	7.125	b	6.877	a
P(F) Treat	*0.000*			
P (F) Year	*0.000*			
*P(F) Treat*Year*	*ns*			
*P(F) Rhizo*Bulk soil*	*0.007*			
	AOA (logcopy DNA $^{-1}$)			
Bacillus subtilis (SM)	6.657	b	7.915	b
Green compost+TW2 (CM)	6.974	a	7.884	b
Green compost (CV)	6.721	b	8.149	a
Trichoderma sp. TW2 (TW2)	6.674	b	7.935	b
Untreated control - C	6.681	b	7.926	b
P(F) Treat	*0.000*			
P (F) Year	*0.000*			
*P(F) Treat*Year*	*0.015*			
*P(F) Rhizo*Bulk soil*	*0.000*			

Table 6 Abundance of *Bacillus subtilis* and *Trichoderma* spp. genes in the rhizosphere and bulk soil after different treatments and the untreated control

Treatment	Rhizosphere soil		Bulk soil	
	Bacillus (logcopy DNA $^{-1}$)			
Bacillus subtilis (SM)	6.024	a	6.458	a
Green compost +TW2 (CM)	4.702	c	5.798	b
Green compost (CV)	5.980	b	5.662	c
Trichoderma sp. TW2 (TW2)	4.673	d	4.865	d
Untreated control - C	4.685	d	4.931	d
P(F) Treat	*0.000*			
P (F) Year	*0.000*			
*P(F) Treat*Year*	*ns*			
*P(F) Rhizo*Bulk soil*	*0.013*			
	Trichoderma (logcopy DNA $^{-1}$)			
Bacillus subtilis (SM)	4.762	d	3.731	c
Green compost+TW2 (CM)	6.922	a	4.483	a
Green compost (CV)	5.494	c	3.652	b
Trichoderma sp. TW2 (TW2)	6.832	b	4.632	a
Untreated control - C	4.791	d	3.977	c
P(F) Treat	*0.000*			
P (F) Year	*0.000*			
*P(F) Treat*Year*	*ns*			
*P(F) Rhizo*Bulk soil*	*0.016*			

916 Table 7 Abundance of the *chiaA* gene in the rhizosphere and bulk soil after different treatments and
917 the untreated control
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Treatment	Rhizosphere soil		Bulk soil	
	chiA gene (logcopy DNA $^{-1}$)			
Bacillus subtilis (SM)	3.632	c	4.384	b
Green compost+TW2 (CM)	5.736	a	4.363	b
Green compost (CV)	4.338	b	4.403	a
Trichoderma sp. TW2 (TW2)	5.789	a	4.321	b
Untreated control - C	3.373	c	3.890	c
P(F) Treat	*0.000*			
P (F) Year	*0.000*			
*P(F) Treat*Year*	*ns*			
*P(F) Rhizo*Bulk soil*	*0.015*			

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Table 8 Pearson's correlation coefficient for the BCAs, Fol race 1 abundance and chemical properties in the rhizosphere (rhizo) and bulk soil samples

Property	*Bacillus subtilis*		*Trichoderma* spp.		Fol	
	rhizo	bulk soil	rhizo	bulk soil	rhizo	bulk soil
pH	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
TN	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
NH_4^+	*ns*	*ns*	0.78***	*ns*	*ns*	*ns*
NO_3^-	-0.5	ns	-0.75***	ns	ns	ns
TP	-0.60***	-0.40**	0.5**	ns	-0.4**	-0.5**
AP	0.40**	-0.50**	0.67**	0.50**	0.575**	0.468*
DOC	-0.7***	0.5 *	-0.8***	0.67***	0.676***	0.67**

Significance levels: not significant-ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$

Table 9 Pearson's correlation coefficients between microbial gene abundance and disease severity (DS) in rhizosphere (rhizo) and bulk soil samples

Microbial abundance	Disease severity (DS)	
	rhizo	bulk soil
Fol	0.75***	0.63***
16S Bacteria	ns	ns
16S Archaea	ns	ns
18S Fungi	0.67	ns
AOB	-0.55**	0.65***
AOA	-0.57***	-0.76***
Bacillus	-0.45**	-0.47*
Trichoderma	-0.75***	-0.60***
chiA gene	-0.55***	-0.65***

Significance levels: not significant-ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$